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(54) **Novel vector plasmids.**

(57) Disclosed is a novel vector plasmid and the process for producing the same by inserting a DNA fragment containing a gene expressible in a microorganism belonging to the genus *Corynebacterium* or *Brevibacterium* into a plasmid derived from a microorganism belonging to the genus *Corynebacterium* or *Brevibacterium*.

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NOVEL VECTOR PLASMIDS

Background of the Invention

The present invention relates to a vector plasmid and the process for producing the same by inserting a DNA fragment containing a gene expressible in a microorganism belonging to the genus Corynebacterium or Brevibacterium into a plasmid derived from a microorganism belonging to the genus Corynebacterium or Brevibacterium. The resultant plasmid facilitates the cloning of desired genes in a host microorganism of the genus Corynebacterium or Brevibacterium.

Genetic engineering technology has made it possible to insert a DNA fragment containing a desired gene into a vector such as plasmids and phages, introduce the thus obtained recombinant DNA into a microbial cell and make the microorganism inherit the gene by the help of autonomous replication of the vector.

Since genetic engineering technology has been established first using Escherichia coli as a host microorganism, such application of the technology as the production of useful metabolites by the fortification of the specific metabolic system and the production of useful proteins by the introduction of a gene derived from eucaryotes has been conducted using Escherichia coli.

Although the knowledge on Escherichia coli accumulated for a long time has greatly contributed to the progress of these researches, the successful development of useful vectors cannot be neglected. The importance of vectors in genetic engineering technology is clearly explained in Recombinant Molecules: Impact on Science and Society, Miles International Symposium Series No. 10, edited by R. F. Beers and E. G. Basset, Raven Press, New York, 1977.

Plasmid vectors are preferred in applied researches using Escherichia coli as host. A typical plasmid vector, pBR322 shows well the advantages of plasmid vectors. pBR322 DNA can be readily recovered because of its replication characteristic of having many copies in a cell and a DNA fragment can be cloned in the plasmid without preventing the replication of pBR322 because of its small molecular weight and only one cleavage site for various restriction endonucleases. Genes responsible for the ampicillin resistance and the tetracycline resistance (hereinafter referred to as "Am^R gene" and "Tc^R gene" respectively) can be used as a marker to select a microorganism containing the plasmid.

The insertional inactivation is an additional advantage of pBR322. It has only one restriction site for each of PstI, BamHI, HindIII and SalI and the PstI site resides in the Am^R gene and the other three reside in the

Tc^R gene. The insertion of a DNA fragment into these restriction sites will result in the loss of resistance due to the cleavage of the gene (insertional inactivation). Thus, it is possible to select a strain having a recombinant DNA by first selecting strains which are resistant to one of the drugs (Am or Tc) and then selecting those which become sensitive to the other drug due to the insertional inactivation [refer to Bolivar, F. et al.: Gene, 2, 95 (1977)]. A lot of practical plasmid vectors for Escherichia coli have been constructed which are similar to pBR322 in characteristics. For example, as a plasmid vector capable of the insertional inactivation of genes responsible for drug-resistance with more restriction endonucleases, pGA22 has been prepared. Since pGA22 has genes responsible for the resistance to chloramphenicol and kanamycin (hereinafter referred to as "Cm^R gene" and Km^R gene" respectively) in addition to Am^R gene and Tc^R gene of pBR322, the insertional inactivation of Cm^R gene at EcoRI site and of Km^R gene at HindIII or XhoI site is possible as well as the insertional inactivation of Am^R gene and Tc^R gene [refer to An, G. et al.: J. Bacteriol., 140, 400 (1979)].

On the other hand, for industrially useful microorganisms other than Escherichia coli, such as amylase-producing Bacillus subtilis, antibiotics-producing Actinomycetes and alcohol-producing yeasts, the recombinant DNA technology has been developed and vectors in these microorganisms have been obtained. Examples of the practical application of the technology using these organisms as host are few. One of the reasons of such limited use is that in these microorganisms such useful plasmid vector as those of Escherichia coli has not been discovered yet. If Escherichia coli plasmids or fragments thereof are joined with plasmids for these organisms, they will automatically aquire the usefulness of Escherichia coli plasmids described above. However, no successful use of drug resistance genes of Escherichia coli plasmids as vector markers has not been known in these species. It has been said that although Gram positive Bacillus subtilis and Actinomycetes are procaryotes,

genes of Gram negative Escherichia coli can not express in these microorganisms. In fact, it has been reported when a gene responsible for drug resistance of Escherichia coli used as a selection marker of Escherichia coli vector plasmid is introduced into Bacillus subtilis or Actinomycetes after recombination with the plasmids of these microorganisms, the recombinant plasmid can replicate but the drug resistance gene can not be expressed [refer to Kreft, J. et al.: Molec. Gen. Genet., 162, 59 (1978), Schottel, J. L. et al.: J. Bacteriol., 146, 360 (1981)]. Therefore, the result obtained in Escherichia coli seems not to be applicable directly to these Gram positive microorganisms.

The present inventors have investigated about the applicability of the result obtained by highly developed genetics and gene engineering technology on Escherichia coli to Gram positive microorganisms of the genera Corynebacterium and Brevibacterium in order to improve efficiently these microorganisms to produce useful substances. As a result, in spite of the accepted theory that genes of Gram negative microorganisms are difficult to express in cells of Gram positive microorganisms, the present inventors have found that the microorganisms of the genus Corynebacterium or Brevibacterium have an ability to express foreign genes of other microorganisms such as Escherichia coli. Thus, the present invention has been completed.

Summary of the Invention

According to the present invention, useful plasmid vectors are provided for recombinant DNA technology using microorganisms of the genera Corynebacterium and Brevibacterium as host microorganisms. The main advantage of the present invention is in facilitating the recombinant DNA technology in the microorganisms of the genera Corynebacterium and Brevibacterium by inserting a gene expressible in cells of Escherichia coli into a plasmid autonomously replicable in cells of Corynebacterium and Brevibacterium to create selective markers and new cleavage sites for restriction endonucleases in the plasmid. The genes to be

inserted into a plasmid include not only genes responsible for drug-resistance derived from Escherichia coli plasmid and genes located on the chromosome of Escherichia coli, but also genes which are derived from other microorganisms than Escherichia coli. Recombinant plasmids wherein said genes are inserted into a plasmid of the genus Corynebacterium or Brevibacterium are constructed by conventional in vitro recombinant DNA technology and introduced into a microorganism of the genus Corynebacterium or Brevibacterium. Desired transformants are selected by the expression of the inserted genes, and the recombinant plasmids are recovered from cultured cells of the selected transformants.

Brief Description of the Drawings

Figs. 1 - 3 show cleavage maps of pCE54, pCB101 and pEthr1 for restriction endonucleases and processes for producing the same. BglII / BamHI with broken line indicates a recombination site at the same cohesive ends formed by cleavage with both restriction endonucleases. Restriction endonucleases used in the preparation of the cleavage map are PstI, KpnI, BamHI, HpaI, EcoRI, SalI and XhoI for pCE54, PstI, EcoRI, HincII and BglII for pCB101, and PstI, EcoRI and XhoI for pEthr1. Molecular weights of the plasmids are indicated with kilobase (Kb).

Fig. 4 shows cleavage map of pCG11.

Detailed Description of the Invention

The present invention provides a vector plasmid and the process for producing the same by inserting a DNA fragment containing a gene expressible in a microorganism belonging to the genus Corynebacterium or Brevibacterium into a plasmid autonomously replicable in cells of a microorganism of the genus Corynebacterium or Brevibacterium. The vector plasmid is autonomously replicable in a microorganism of the genus Corynebacterium or Brevibacterium and the presence of which is detected by the expression of the inserted gene.

As the genes expressible in the microorganism of the genus Corynebacterium or Brevibacterium, those derived from either eucaryotes or procaryotes may be employed. Preferably the genes responsible for drug resistance, the genes responsible for the biosynthesis of cell components such as amino acids, nucleic acids and vitamines, the genes responsible for the assimilation of substrates such as sugars, and the genes of plasmids and phages, derived from coryneform bacteria such as the genus Corynebacterium or Brevibacterium, bacteria belonging to the genera Escherichia, Microbacterium, Staphylococcus, Streptococcus, Pseudomonas, Serratia and Bacillus, yeasts and Actinomycetes are employed.

Examples of the plasmids and phages are pBR322, pBR325, pGA22, pACYC177, pACYC184, and λgtWESλB derived from microorganisms of the genus Escherichia, pUB110, pC194, pTP4 and ø11 derived from the genus Bacillus or Staphylococcus, pCG4 derived from the genus Corynebacterium or

Brevibacterium, pSLP1.2, pSLP111 and SCP2 derived from Actinomycetes and YEp13, YRp7 and YTp1 derived from yeasts.

As the plasmids autonomously replicable in cells of the genus Corynebacterium or Brevibacterium, plasmids pCG1, pCG2, pCG4 and the like are preferably used. These plasmids have been found first by the present inventors and disclosed in Japanese Published Unexamined Patent Application Nos. 18101/81, 133557/81 and 58186/81. Strains having these plasmids have been deposited with the American Type Culture Collection and the Fermentation Research Institute, Agency of Industrial Science and Technology under accession numbers mentioned in Examples below.

The recombinant plasmid vectors of the present invention include any recombinant plasmid vectors of two members selected from genes and plasmids which are obtained from mutually heteromicroorganisms, for example, a recombinant plasmid pCE54 wherein Escherichia coli plasmid pGA22 is combined with plasmid pCG2, a recombinant plasmid pCG11 wherein a part of plasmid pCG4 is combined with plasmid pCG1, recombinant plasmid pCB101 wherein Staphylococcal plasmid pUB110 is combined with plasmid pCG11 and recombinant plasmid pEthr1 wherein Escherichia coli plasmid pGH2 having threonine operon is combined with plasmid pCG11. Each of these plasmids is explained in detail below.

pCE54

pCE54 is prepared as follows.

pCG2 is isolated from cultured cells of Corynebacterium glutamicum 225-218 by the method described in the Japanese patent application mentioned above, and shown in Example 1, and pGA22 is isolated from cultured cells of Escherichia coli in a conventional manner. Both plasmid DNAs are linearized by complete digestion with a restriction enzyme which has only one cleavage site in each plasmid DNA, for example, PstI and treated with T4 phage DNA ligase to form composite molecules wherein cohesive ends of the DNAs are combined with each other. The desired recombinant

plasmid which contains both DNAs are obtained by first selecting transformants of the genus Corynebacterium or Brevibacterium with respect to the drug-resistance derived from pGA22 and then analyzing plasmids in the transformants.

The transformation with the DNA mixture is carried out by the transformation method using protoplasts of cells of the genus Corynebacterium or Brevibacterium, which is described in Japanese Patent Application Nos. 58187/81 and 65777/81 by the present inventors, and described in Example below. Drugs used for the selection are tetracycline, chloramphenicol and kanamycin, to which pGA22 carry drug resistant genes. Ampicillin cannot be used because Am^R gene is inactivated by the insertion of pCG2 at PstI site. Transformants are recovered by isolating the colonies regenerating on a hypertensive agar medium containing a drug in a concentration wherein recipient protoplasts cannot be reversed to normal cells without addition of DNA, generally, 0.4 to 1.6 µg/ml tetracycline, 2.5 to 5 µg/ml chloramphenicol or 100 to 800 µg/ml kanamycin, or by collecting all the colonies regenerating unselectively on a regeneration medium and then isolating the desired colonies grown on an agar medium containing a drug in a concentration wherein normal cells cannot grow, generally, 0.5 to 4 µg/ml tetracycline, 2 to 15 µg/ml chloramphenicol or 2 to 25 µg/ml kanamycin.

Some of transformants selected with respect to the resistance to tetracycline, chloramphenicol or kanamycin possess phenotypes of resistance to other drugs derived from pGA22.

Plasmid DNAs in the transformants can be isolated from cultured cells of the transformants by the method described in Japanese Patent Application Nos. 18101/81 and 65777/81 and in Example below. Structures of the plasmid DNAs can be determined by analyzing the DNA fragments by agarose gel electrophoresis after digestion with various restriction enzymes. pCE54 is isolated from one of the transformants selected as mentioned above. Fig. 1 illustrates the process for producing pCE54 and the cleavage map for various restriction endonucleases, which shows that pCE54

is a composite plasmid of pCG2 and pGA22 combined at their PstI cleavage sites. On the other hand, a composite plasmid of pCG2 and pGA22 wherein the direction of the combination is opposite to that of pCE54 is obtained from another transformant. Transformants resistant to the three drugs can be obtained by transforming microorganisms of the genus Corynebacterium or Brevibacterium with either of the composite plasmids and selecting in the same manner as described above. It has been confirmed that these transformants have the same plasmid as the donor plasmid.

Sensitivities to tetracycline, chloramphenicol and kanamycin of Corynebacterium glutamicum LA103, a derivative of L-22, and a pCE54-containing strain thereof are illustrated in Table 1 as the minimum inhibitory concentration which prevents the growth of 10^4 cells incubated on an NB agar medium (pH 7.2) consisting of 20 g/l powdered bouillon, 5 g/l yeast extract and 18 g/l agar at 30°C for 2 days.

Table 1

Microorganism	Minimum inhibitory concentration (MIC, µg/ml)		
	tetracycline	chloramphenicol	kanamycin
Corynebacterium glutamicum LA 103	0.8	1.6	0.8
Corynebacterium glutamicum LA 103/pCE 54	3.2	12.5	> 400
Corynebacterium glutamicum LA 103/pCB 101	0.8	1.6	200

As apparent from Table 1, the strain containing pCE54 expresses the resistance to the three drugs coded for by genes carried by pGA22. Therefore, pCE54 can be used as effectively in microorganisms of the genus Corynebacterium or Brevibacterium as pGA22 in Escherichia coli, to clone

desired DNA fragments in these microorganisms.

pCG11

pCG11 is a plasmid invented by the present inventors and described in Japanese Patent Application No. 18101/81. pCG11 is constructed by inserting a BamHI fragment of pCG4 isolated from Corynebacterium glutamicum 225-250 (ATCC 31830, FERM P-5939) carrying a gene responsible for the resistance to streptomycin and/or spectinomycin at the unique BglII site of pCG1 isolated from Corynebacterium glutamicum 225-57 (ATCC 31808, FERM P-5865).

pCB101

As mentioned above, genes of Escherichia coli are not expressed in Bacillus subtilis. To the contrary, some of the genes expressed in the microorganisms of the genus Bacillus are known to be expressed in Escherichia coli. For example, it has been reported that when Escherichia coli is transformed with recombinant plasmids of a plasmid of Escherichia coli and a DNA carrying the genes responsible for drug resistance derived from either plasmid or chromosome of Gram positive bacteria such as genus Bacillus, these drug resistance genes are expressed [refer to Ehrlich, S.D.: Proc. Natl. Acad. Sci., USA, 75, 1433 (1978), Kreft, J. et al.: Molec. Gen. Genet., 162, 59 (1978) and Gray, O. et al.: J. Bacteriol, 145, 422 (1981)]. Therefore, genes of the microorganisms other than Escherichia coli can be used to construct plasmid vectors for the microorganisms of the genus Corynebacterium or Brevibacterium as useful as pCE54. A derivative plasmid wherein the characteristic of plasmid pUB110 is introduced is explained below as an example of such plasmid vectors.

The plasmid pUB110 was originally isolated from a microorganism of the genus Staphylococcus resistant to kanamycin or neomycin. pUB110 is employed as a vector in Bacillus subtilis since it can replicate in Bacillus subtilis and its gene responsible for the resistance to kanamycin or neomycin can be expressed in Bacillus subtilis [refer to

Keggins, K. M. et al.: Proc. Natl. Acad. Sci., USA, 75, 1423 (1978)]. The expression of Km^R gene of pUB110 in Escherichia coli is confirmed by measuring the degree of the resistance to kanamycin of Escherichia coli strain containing a recombinant plasmid of pUB110 and an Escherichia coli plasmid prepared by recombinant DNA technology. pUB110 and an Escherichia coli plasmid pBR325 [refer to Bolivar, F. et al.: Gene, 4, 121 (1978)] are each cleaved and ligated at the BamHI site to obtain two types of recombinant plasmids wherein the directions of combination are opposite to each other. The minimum inhibitory concentration of kanamycin is 50 µg/ml against Escherichia coli K-12 C 600 strains containing either type of the plasmid. Since the minimum inhibitory concentration of kanamycin against C 600 strain which does not contain the plasmid is 1.6 µg/ml, it is obvious that the Km^R gene of pUB110 is expressed in Escherichia coli.

pCB101 prepared by combining pUB110 and pCG11 is an example of application of the usefulness of pUB110 to plasmids for genus Corynebacterium or Brevibacterium.

pCB101 can be prepared as follows.

pCG11 isolated from Corynebacterium glutamicum LA 103 is digested with BglII and pUB110 isolated from Bacillus subtilis is digested with BamHI to linearize them. Since both plasmids have the same cohesive ends due to the cleavage specificity of BglII and BamHI, treatment of the mixture of two digests with T4 phage ligase gives various recombinant plasmids formed through the complementary base pairing with the cohesive ends. The mixture is used to transform the protoplast of Corynebacterium glutamicum LA 103. Transformants can be selected with respect to the resistance to kanamycin derived from pUB110 in the same manner as in the selection of pCE54. Some transformants resistant to kanamycin get simultaneously the spectinomycin resistance encoded for by pCG11.

The structure of plasmid DNA in the strain resistant to both kanamycin and spectinomycin is determined by digesting the plasmid DNA with various restriction

endonucleases and analyzing the fragments by agarose gel electrophoresis. pCB101 is a plasmid isolated from one of the transformants. The process for producing pCB101 and the cleavage map thereof are illustrated in Fig. 2. As is apparent from Fig. 2, pCB101 is a composite plasmid wherein BglII-opened pCG11 is inserted at the BamHI site of pUB110. Another transformant gives a composite plasmid wherein the orientation of the insertion of pCG11 and pUB110 is opposite to that of pCB101. When either composite plasmid is introduced into Corynebacterium glutamicum LA 103 in the same manner as mentioned above and transformants are selected with kanamycin or spectinomycin, the transformants also gain resistance to the other drug which is not used for selection and have the same plasmids as donor plasmids characterized by cleavage sites for various restriction endonucleases.

Minimum inhibitory concentrations of kanamycin against Corynebacterium glutamicum LA 103 with or without pCB101 are illustrated in Table 1. As is apparent from Table 1, the strain with pCB101 expresses not only the spectinomycin resistance gene derived from pCG11, but also the Km^R gene derived from pUB110. Thus pCB101 has two drug-resistance genes which will serve as selective markers in the microorganisms of the genus Corynebacterium or Brevibacterium. In addition, pCB101 will facilitate cloning of desired DNA fragments in these microorganisms by insertional inactivation, since there is a BglII site in the Km^R gene in pUB110 [refer to Gryczan, T. et al.: J. Bacteriol., 141, 246 (1980)].

pEthr1

As explained above, it is possible to make plasmids of the microorganisms of the genus Corynebacterium or Brevibacterium more useful by attaching the drug resistance genes expressible in Escherichia coli as vector marker. Useful plasmid vectors can also be prepared by inserting chromosomal genes expressible in Escherichia coli, for example, genes responsible for metabolism. Since the role of a vector

marker is to facilitate the selection of the strain having recombinants, when the host microorganism is a mutant lacking a certain phenotype, a plasmid having a chromosomal gene of a wild type will serve as useful vector with selection marker in a mutant.

Plasmid vectors carrying chromosomal genes as selection marker in Corynebacterium or Brevibacterium can be constructed by conventional in vitro recombinant DNA technology as described above. A fragment of chromosomal DNA extracted from a microorganism or a DNA fragment cloned in an Escherichia coli vector is inserted in vitro in a plasmid of the microorganism of the genus Corynebacterium or Brevibacterium to make a recombinant plasmid. Then, a mutant strain belonging to the genus Corynebacterium or Brevibacterium and lacking a certain phenotype is transformed with the recombinant plasmid, followed by the selection of a transformant wherein the lost phenotype is complemented. The mutant lacking a certain phenotype can be obtained by a conventional mutation method.

For example, a derivative plasmid wherein a DNA fragment containing an Escherichia coli threonine operon is inserted in a plasmid of the microorganism of the genus Corynebacterium is used as a vector in a host microorganism belonging to Corynebacterium glutamicum and requiring homoserine or methionine plus threonine. The threonine operon of Escherichia coli in the plasmid can serve as a selection marker because the requirement for homoserine due to the lack of homoserine dehydrogenase in the host strain is complemented with the homoserine dehydrogenase gene coded in the threonine operon of Escherichia coli.

A plasmid pEthr1 containing a threonine operon of Escherichia coli is explained below.

A DNA fragment containing a threonine operon of Escherichia coli is cloned using a host-vector system of Escherichia coli. A chromosomal DNA extracted from an Escherichia coli strain having a wild type threonine operon and an Escherichia coli vector plasmid pGA22 are digested with a restriction endonuclease, HindIII. The mixture of

the two digests is treated with T4 phage ligase. Escherichia coli K-12, GT-3 requiring homoserine and diaminopimelic acid is transformed with the mixture in a conventional manner and transformants grown on a minimum medium containing kanamycin and diaminopimelic acid are selected. Plasmids in the transformants are isolated from cultured cells in a conventional manner and the structure is determined by analyzing DNA fragments formed by digestion with various restriction endonucleases by agarose gel electrophoresis. One of the thus obtained plasmids is pGH2 illustrated in Fig. 3. A DNA fragment containing an Escherichia coli threonine operon has already been cloned and the cleavage map was determined [refer to Cossart, P. et al.; Molec. Gen. Genet., 175, 39 (1979)]. It is certain that pGH2 has a threonine operon because the inserted fragment has the same restriction map as that of Escherichia coli threonine operon.

pEthr1 is constructed as a recombinant of pGH2 and pCG11. pGH2 is digested with BamHI and pCG11 is digested with BglII in a conventional manner. The mixture of the digests is treated with T4 ligase. Protoplasts of Corynebacterium glutamicum LA 201, a mutant requiring homoserine and leucine and derived from L-22 strain, are transformed with the mixture in the same manner as described above. The protoplasts are unselectively regenerated on a regeneration medium. Then, the regenerated cells are collected and spread on a minimum medium containing leucine and the formed colonies are recovered. Some of the thus obtained strains which do not require homoserine have both phenotype of kanamycin-resistance derived from pGH2 and that of spectinomycin-resistance derived from pCG11. pEthr1 is a plasmid isolated from one of such transformants. The process for the construction of pEthr1 and the cleavage map of pEthr1 based on the analysis by agarose gel electrophoresis after the digestion with various endonucleases are illustrated in Fig. 3. It is apparent from Fig. 3 that pEthr1 is a plasmid wherein a fragment containing a threonine operon of pGH2 cleaved with BamHI is inserted in pCG11. From another transformant, a plasmid wherein the orientation

of the BamHI fragment of pGH2 is opposite to that in pEthr1 is obtained. The threonine operon inserted at either orientation can complement homoserine-requirement in Corynebacterium glutamicum LA 201 which has become resistant to kanamycin and spectinomycin. The thus obtained transformants have the donor plasmid characterized by the cleavage sites for various restriction endonucleases. Therefore, pEthr1 is a plasmid which facilitates cloning of DNA fragments in Corynebacterium glutamicum LA 201 because the homoserine dehydrogenase gene on the threonine operon derived from Escherichia coli serves as a selection marker.

The thus prepared plasmids for Corynebacterium or Brevibacterium containing a selection marker can be used as a vector for cloning a DNA fragment carrying desired genes in a host microorganism belonging to the genus Corynebacterium or Brevibacterium. Cloning is carried out by recombining a donor DNA and a vector plasmid by in vitro DNA recombination technology and transforming the microorganism of the genus Corynebacterium or Brevibacterium with the recombinant plasmid in a conventional manner as described above. Especially, when the donor DNA is inserted into a cleavage site present in the gene introduced as a vector marker, the desired recombinants are easily selected by checking the loss of phenotypic expression of the gene in the transformants.

The above-described plasmids pCE54, pCG11, pCB101 and pEthr1 introduced in Corynebacterium glutamicum LA 103 or LA 201 have been deposited with the American Type Culture Collection in USA under accession numbers shown in Table 2.

Table 2

Strain (host microorganism/plasmid)	ATCC No.
Corynebacterium glutamicum LA103/pCE54	39019
Corynebacterium glutamicum LA103/pCG11	39022
Corynebacterium glutamicum LA103/pCB101	39020
Corynebacterium glutamicum LA201/pEthr1	39021

In the three plasmids, the Escherichia coli gene or the gene expressible in cells of Escherichia coli is merely inserted into the plasmid of Corynebacterium glutamicum without using special technique for expression. As the genes of the DNA fragments inserted at any orientation into the plasmid of Corynebacterium glutamicum are expressed in Corynebacterium glutamicum, it is apparent that Corynebacterium glutamicum has the ability to recognize precisely the initiation signals for transcription and translation of the introduced gene to accomplish the transcription and translation. In view of the presence of common base sequences required for precise transcription and translation in Escherichia coli genes, Corynebacterium is considered to be able to recognize the initiation sites for transcription and translation of other Escherichia coli genes than those described above and to express the genes. Therefore, any genes expressible in the microorganism of Escherichia coli can be introduced as the marker into the plasmids of the present invention.

Since the usefulness of the present invention is in providing a vector plasmid to the microorganisms of the genus Corynebacterium or Brevibacterium having a selection marker, the purpose of the present invention is achieved by inserting a marker gene into a plasmid of the microorganism of the genus Corynebacterium or Brevibacterium in such a way as to be expressed. Therefore, in the case of the genes used in the present specification, the method of inserting genes into the plasmids of Corynebacterium glutamicum is not restricted to those described in Examples. Since any plasmid autonomously replicable in the microorganisms of the genus Corynebacterium or Brevibacterium can be used to make a plasmid having a selection marker by inserting a gene expressible in Escherichia coli, plasmids of the microorganism of the genus Corynebacterium or Brevibacterium are not restricted to those described in the present specification.

In spite of many common microbial properties, microorganisms with high glutamic acid productivity (so

called glutamic acid-producing microorganisms) are classified to various species and even genera such as Corynebacterium and Brevibacterium probably because of their industrial importance. However, it has been pointed out that these microorganisms should be classified to one species because they have homology in the amino acids in the cell walls and the GC content of DNA. Recently, it has been reported that these microorganisms have more than 70% homology in DNA, indicating that these microorganisms are very closely related [refer to Komatsu, Y.: Report of the Fermentative Research Institute, No. 55, 1 (1980), and Suzuki, K., Kaneko, T., and Komagata, K.: Int. J. Syst. Bacteriol., 31, 131 (1981)].

In the present specification, the usefulness of the plasmid of the present invention is shown using derivatives of Corynebacterium glutamicum L-22 as host microorganisms because of the regulation on the experiments using recombinant DNA technology. However, in consideration of the fact mentioned above, it is apparent that the usefulness of the plasmid of the present invention is applicable to all the glutamic acid-producing microorganisms. In order to use the plasmid as a vector in these microorganisms, slight difference in the properties of host microorganisms such as homology of the DNA is negligible and it is sufficient that these microorganisms have functions for the autonomous replication of the plasmids and the expression of introduced genes. It is apparent that these microorganisms have such functions from the fact that plasmid pCG4 which is isolated from Corynebacterium glutamicum 225-250 and has a streptomycin and/or spectinomycin resistance gene (Japanese Patent Application No. 58186/81) can replicate in glutamic acid-producing microorganism belonging to the genus Corynebacterium or Brevibacterium and the streptomycin and/or spectinomycin resistance gene can be expressed. Therefore, the host of the plasmids of the present invention is not limited to Corynebacterium glutamicum but includes all the glutamic acid-producing microorganisms including the microorganisms belonging to the genus Corynebacterium or Brevibacterium.

Examples of the present invention are as follows.

Example 1

Preparation of pCE54:

(1) Isolation of pCG2 and pGA22:

Corynebacterium glutamicum 225-218 strain (FERM P-5954, ATCC 31832) is cultured with shaking at 30°C for 18 hours in NB medium (pH 7.2) consisting of 20 g/l powdered bouillon and 5 g/l yeast extract. 5 ml of the culture is inoculated to 400 ml of a semisynthetic medium SSM (pH 7.2) consisting of 20 g/l glucose, 10 g/l $(NH_4)_2SO_4$, 3 g/l urea, 1 g/l yeast extract, 1 g/l KH_2PO_4, 0.4 g/l $MgCl_2 \cdot 6H_2O$, 10 mg/l $FeSO_4 \cdot 7H_2O$, 0.2 mg/l $MnSO_4 \cdot (4-6)H_2O$, 0.9 mg/l $ZnSO_4 \cdot 7H_2O$, 0.4 mg/l $CuSO_4 \cdot 5H_2O$, 0.09 mg/l $Na_2B_4O_7 \cdot 10H_2O$, 0.04 mg/l $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 30 µg/l biotin and 1 mg/l thiamine·HCl and culturing is carried out with shaking at 30°C. Optical density (OD) of the culture medium is measured at 660 nm with a Tokyo Koden Colorimeter and at an OD value of 0.2 penicillin G is added to a concentration of 0.5 U/ml. Culturing is continued at 30°C to an OD value of about 0.6.

Cells are recovered from the culture medium and washed with TES buffer solution (pH 8.0) consisting of 0.03M tris (hydroxymethyl) aminomethane (referred to as "Tris" hereinafter), 0.005M EDTA, and 0.05M NaCl. The washed cells are suspended in a lysozyme solution (pH 8.0) consisting of 25% sucrose, 0.1M NaCl, 0.05M Tris and 0.8 mg/ml lysozyme to make 10 ml of a suspension. The suspension is allowed to react at 37°C for 4 hours. 2.4 ml of 5M NaCl, 0.6 ml of 0.5M EDTA (pH 8.5) and 4.4 ml of 4% sodium lauryl sulfate and 0.7M NaCl solutions are added to the suspension successively. The mixture is shaken slowly and put on an ice water for 15 hours. The whole lysate is transferred into a centrifugation tube and centrifuged at 4°C at 69,400 x g for 60 minutes to recover the supernatant fluid. Polyethyleneglycol (PEG 6000) (product of Nakarai Kagaku Yakuhin Co.) is added to 10% by weight, and the mixture is shaken slowly and put on an ice water for 10 hours. The mixture is centrifuged at 1,500 x g for 10 minutes to recover a pellet. The pellet is dissolved gently in 5 ml of TES

buffer solution. 2.0 ml of 1.5 mg/ml ethidium bromide solution is added and thereafter cesium chloride is added to adjust the density to 1.580. The solution is subjected to density gradient centrifugation at 105,000 x g at 18°C for 48 hours. A covalently closed circular DNA is detected by UV irradiation as a high density band located in the lower part of the centrifugation tube. The band is taken out from the side of the centrifugation tube with a syringe to obtain a fraction containing plasmid pCG2. Then, the fraction is treated five times with an equal amount of cesium chloride saturated isopropyl alcohol solution consisting of 90% by volume isopropyl alcohol and 10% TES buffer solution to extract and remove ethidium bromide. The residue is subjected to dialysis against TES buffer solution to obtain 40 µg of pCG2 plasmid DNA.

pGA22 is isolated from cultured cells of an Escherichia coli K-12 derivative, prepared by An, G. et al. according to the method of An, et al. [J. Bacteriol., 140, 400 (1979)].

(2) In vitro recombination of pCG2 and pGA22

4 units of PstI (product of Takara Shuzo Co., 6 units/µl) is added to 200 µl of a restriction endonuclease reaction solution consisting of 20 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 0.01% bovine serum albumin (pH 7.5) and 2 µg each of pCG2 and pGA22 plasmid DNAs prepared as above. The mixture is allowed to react at 30°C for 60 minutes and then heated at 65°C for 10 minutes to stop the reaction. The linearization of the both circular plasmids is confirmed by analyzing the sample treated in the same manner by horizontal 0.8% agarose gel electrophoresis containing 0.6 µg/ml ethidium bromide.

40 µl of T4 ligase buffer solution (pH 7.6) consisting of 660 mM Tris, 66 mM $MgCl_2$ and 100 mM dithiothreitol, 40 µl of 5 mM ATP, 0.2 µl of T4 ligase (product of Takara Shuzo Co., 1 unit/µl) and 120 µl of H_2O are added to 200 µl of the reaction mixture. The mixture is allowed to react at 12°C for 16 hours and extracted twice with 400 µl of

phenol saturated with TES buffer solution. The residue is subjected to dialysis against TES buffer solution to remove phenol.

(3) Recovery of pCES4

Transformation is carried out using the protoplast of recipient cells. A seed culture of Corynebacterium glutamicum LA 103 is inoculated in the NB medium and cultured with shaking at 30°C. Cultured cells are collected at an OD value of 0.6 and suspended in an RCGP medium (pH 7.6) containing 1 mg/ml lysozyme at a concentration of about 10^9 cells/ml. The RCGP medium consists of 5 g/l glucose, 5 g/l casamino acid, 2.5 g/l yeast extract, 3.5 g/l K_2HPO_4, 1.5 g/l KH_2PO_4, 0.41 g/l $MgCl_2 \cdot 6H_2O$, 10 mg/l $FeSO_4 \cdot 7H_2O$, 2 mg/l $MnSO_4 \cdot (4-6)H_2O$, 0.9 mg/l $ZnSO_4 \cdot 7H_2O$, 0.04 mg/l $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 30 µg/l biotin, 2 mg/l thiamine hydrochloride, 135 g/l sodium succinate and 30 g/l polyvinylpyrrolidone of a molecular weight of 10,000. The suspension is put into an L-tube and allowed to react with gentle shaking at 30°C for 5 hours to make protoplasts.

0.5 ml of the protoplast suspension is transferred into a small tube and subjected to centrifugation at 2,500 x g for 5 minutes. The residue is resuspended in 1 ml of a TSMC buffer solution (pH 7.5) consisting of 10 mM magnesium chloride, 30 mM calcium chloride, 50 mM Tris and 400 mM sucrose and subjected to centrifugation and washing. The residue is resuspended in 0.1 ml of the TSMC buffer solution. 100 µl of a mixture of a two-fold concentrated TSMC buffer solution and the above-described DNA mixture treated with ligase (1 : 1) is added to the suspension and 0.8 ml of a TSMC buffer solution containing 20% PEG 6,000 is added. After 3 minutes, 2 ml of the RCGP medium (pH 7.2) is added and the mixture is subjected to centrifugation at 2,500 x g for 5 minutes. The supernatant fluid is removed and the precipitated protoplasts are suspended in 1 ml of the RCGP medium. The suspension is slowly shaken at 30°C for 2 hours to express the gene.

Thereafter, a suitable amount of the protoplast suspension

is spread on an RCGP agar medium (pH 7.2) containing 400 µg/ml kanamycin and 1.4% agar and cultured at 30°C for 6 days.

Five of the kanamycin-resistant transformants are selected at random and purified on NB agar medium containing 12.5 µg/ml kanamycin. The purified five strains are propagated in 400 ml of the NB medium to an OD value of about 0.8 and then cells are collected. Plasmids are isolated from the cells by the same process as described in the process (1) to isolate pCG2. 45 - 55 µg of plasmid DNA is obtained from each transformant. 0.5 µg of the plasmid DNA is single- or double-digested with various restriction endonucleases and the DNA fragments formed are analyzed by agarose gel electrophoresis to determine molecular weights of the fragments and the cleavage sites in the plasmid molecules. The restriction endonucleases such as HpaI, PstI, KpnI, BamHI, EcoRI, SalI and XhoI (HpaI is a product of Boehringer Mannheim GmbH and the other enzymes are products of Takara Shuzo Co.) are used. The molecular weight is determined by reference to the standard curve plotted with electrophoretic distances of the HindIII fragments of known molecular weight derived from λphage DNA. All of the five plasmids have the structure of a composite plasmid of pCG2 and pGA22. Two of them have the structure illustrated as pCE54 in Fig. 1 and the others have the structure wherein the orientation of the combination of pCG2 with pGA22 is opposite. Transformants having either of the plasmids are endowed with the resistance to tetracycline, chloramphenicol and kanamycin derived from pGA22 as is the case shown in Table 1.

Corynebacterium glutamicum LA 103 transformed with these plasmid DNAs by the same method as above is also endowed with the resistance to the three drugs and has the same plasmid as the donor plasmid identified with the cleavage pattern for various restriction endonucleases.

Example 2

Preparation of pCG11:

pCG1 is isolated from Corynebacterium glutamicum 225-57 by the method used for the isolation of pCG4 from Corynebacterium glutamicum 225-250. Plasmid pCG1 is completely digested with BglII which is a restriction endonuclease derived from Bacillus globigii (product of Takara Shuzo Co.) and plasmid pCG4 is completely digested with BamHI (product of Takara Shuzo Co.) under suitable conditions for each restriction endonuclease. 0.1 unit of T4 phage DNA ligase (product of Takara Shuzo Co.) is mixed with 0.2 ml of a ligase reaction solution (pH 7.6) consisting of 66 mM Tris-HCl, 6.6 mM $MgCl_2$, 10 mM dithiothreitol and 0.5 mM ATP and containing 0.5 µg each of both digests and the mixture is allowed to react at 4°C overnight. The protoplast of Corynebacterium glutamicum LA 103 strain is transformed with the ligation mixture.

The protoplast of Corynebacterium glutamicum LA 103 strain is prepared as in Example 1. The transformation and selection of transformants are carried out by the same method as in Example 1. For transformation, 0.1 ml of the reaction mixture is used. 50 µg of a plasmid DNA is isolated from one of the spectinomycin-resistant transformants by the same method as in Example 1. The plasmid DNA is subjected to single and double digestion with various restriction endonucleases. The resultant DNA fragments are analyzed by the same agarose gel electrophoresis as in Example 1 to determine the molecular weight and the cleavage sites in the plasmid molecule. The cleavage map of the plasmid pCG11 is illustrated in Fig. 4.

Corynebacterium glutamicum LA 103 is transformed with pCG11 plasmid DNA in the same manner as above. The resultant spectinomycin-resistant strain has a plasmid characterized by the same cleavage pattern as that of pCG11.

Example 3

Preparation of pCB101:

(1) Isolation of pCG11 and pUB110:

Corynebacterium glutamicum LA 103/pCG11 (ATCC 39022) is grown in 400 ml of the NB medium to an OD value of about 0.8 and pCG11 is isolated from cultured cells by the same method as in the isolation of pCG2 in Example 1 (1).

pUB110 is isolated from cultured cells of Bacillus subtilis BR 151/pUB110 [Proc. Natl. Acad. Sci. USA, 75, 1423 (1978)] by the method of Gryczan, et al. [refer to Gryczan, T. J., et al.: J. Bacteriol., 134, 318 (1978)].

(2) In vitro recombination of pCG11 and pUB110:

2 units of BglII (product of Takara Shuzo Co., 6 units/µl) is added to 100 µl of the BglII reaction buffer solution (pH 7.5) consisting of 10 mM Tris-HCl, 7 mM $MgCl_2$, 60 mM NaCl and 7 mM 2-mercaptoethanol and containing 2 µg of pCG11 plasmid DNA prepared as above. The mixture is allowed to react at 37°C for 60 minutes. Separately, 2 units of BamHI (product of Takara Shuzo Co., 6 units/µl) is added to 100 µl of the BamHI reaction buffer solution (pH 8.0) consisting of 10 mM Tris-HCl, 7 mM $MgCl_2$, 100 mM NaCl, 2 mM mercaptoethanol and 0.01% bovine serum albumin and containing 2 µg of pUB110 plasmid DNA. The mixture is allowed to react at 37°C for 60 minutes.

Both digests are mixed and 40 µl of the T4 ligase buffer solution, 40 µl of ATP (5 mM), 0.2 µl of T4 ligase and 120 µl of H_2O are added. The mixture is allowed to react at 12°C for 16 hours. The mixture is extracted twice with 400 µl of phenol saturated with TES buffer solution and the extract is subjected to dialysis against TES buffer solution to remove phenol.

(3) Recovery of pCB101

Corynebacterium glutamicum LA 103 is transformed using 100 µl of a mixture (1 : 1) of a two-fold concentrated TSMC buffer solution and the ligase reaction mixture mentioned above as a donor DNA in the same manner as in Example

1 (3) and kanamycin-resistant strains are selected. The formed colonies are replica-plated to an NB agar medium containing 12.5 µg/ml kanamycin and 100 µg/ml spectinomycin. Culturing is carried out at 30°C for 2 days. Three strains resistant to both drugs are selected at random and purified on the same agar medium. The three purified strains are grown in 400 µl of the NB medium to an OD value of about 0.8. Cells are recovered and the plasmids are isolated from the cells by ethidium bromide-cesium chloride density gradient centrifugation described in Example 1 (1). 30 - 35 µg of plasmid DNA is obtained from each transformant.

These plasmid DNAs are digested with restriction endonuclease and analyzed by agarose gel electrophoresis as in Example 1 (3) to determine the molecular weights and cleavage map for restriction endonucleases PstI, EcoRI, HincII and BglII. All the plasmids of the three strains have the structure of a composite plasmid of pCG11 and pUB110. Two of them have the structure illustrated as pCB101 in Fig. 2 and the other has the structure wherein the orientation of the combination of pCG11 and pUB101 is opposite.

Transformants having either of the plasmids are endowed with the phenotype of the resistance to spectinomycin derived from pCG11 and that of the resistance to kanamycin from pUB110.

Corynebacterium glutamicum LA 103 is retransformed with these plasmid DNAs. The resultant kanamycin-resistant transformant is endowed simultaneously with the phenotype of the resistance to spectinomycin. It has the same plasmid as the donor plasmid as characterized by the cleavage pattern for various restriction endonucleases.

Example 4

Preparation of pEthr1:

(1) Cloning of a DNA fragment containing Escherichia coli threonine operon:

Cloning is carried out using a host-vector system of Escherichia coli. pGA22 used as a vector is isolated as in Example 1 (1). A high molecular weight chromosomal DNA used as a donor DNA is isolated from the cultured cells of Escherichia coli K 12 Hfr (ATCC 23740) by the phenol-extraction method of Smith [Smith, M.G.: Method in Enzymology, 12, part A, 545 (1967)]. 0.4 unit of HindIII (product of Takara Shuzo Co., 6 units/µl) is added to 60 µl of a HindIII reaction solution (pH 7.5) consisting of 10 mM Tris-HCl, 7 mM $MgCl_2$ and 60 mM NaCl and containing 4 µg of pGA22 plasmid DNA. The mixture is allowed to react at 37°C for 30 minutes and heated at 65°C for 10 minutes to stop the reaction. pGA22 plasmid DNA is digested with HindIII under the same conditions as above and subjected to agarose gel electrophoresis. It is confirmed that one of the two HindIII cleavage sites present in pGA22 is cleaved.

Separately, 4 units of HindIII is added to 140 µl of the HindIII reaction solution containing 8 µg of the chromosomal DNA. The mixture is allowed to react at 37°C for 60 minutes and heated at 65°C for 10 minutes to stop the reaction.

40 µl of the T4 ligase buffer solution, 40 µl of ATP (5 mM), 0.3 µl of T4 ligase and 120 µl of H_2O are added to a mixture of the digests and the reaction is carried out at 12°C for 16 hours. The reaction mixture is extracted twice with 400 µl of phenol saturated with TES buffer solution and subjected to dialysis against TES buffer solution to remove phenol.

The ligase reaction mixture is used to transform Escherichia coli GT-3 [J. Bacteriol. 117, 133-143 (1974)] which is a derivative of Escherichia coli K-12 and requiring homoserine and diaminopimelic acid. Competent cells of GT-3 strain are prepared according to the method of Dagert, et al. [Dagert, M. et al.: Gene, 6, 23 (1979)]. That is, the strain is inoculated in 50 ml of L-medium (pH 7.2) consisting of 10 g/l Bacto-tryptone, 5 g/l yeast extract, 1 g/l glucose and 5 g/l sodium chloride and containing

100 µg/ml diaminopimelic acid and cultured at 37°C to an OD value of 0.5. The culture is cooled with ice water for 10 minutes and cells are recovered by centrifugation. The cells are suspended in 20 ml of cooled 0.1M calcium chloride. The suspension is allowed to stand at 0°C for 20 minutes and subjected to centrifugation to recover the cells. The cells are suspended in 0.5 ml of 0.1M calcium chloride and allowed to stand at 0°C for 18 hours.

200 µl of the ligase reaction mixture mentioned above is added to 400 µl of the cell suspension treated with calcium chloride. The mixture is allowed to stand at 0°C for 10 minutes and heated at 37°C for 5 minutes. 9 ml of the L-medium is added and the mixture is incubated with shaking at 37°C for 2 hours. Cells are recovered by centrifugation and washed with a physiological saline solution twice. The cells are spread on M9 minimum agar medium (pH 7.2) consisting of 2 g/l glucose, 1 g/l NH_4Cl, 6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.1 g/l $MgSO_4 \cdot 7H_2O$, 15 mg/l $CaCl_2 \cdot 2H_2O$, 4 mg/l thiamine hydrochloride and 15 g/l agar and containing 12.5 µg/ml kanamycin. Culturing is carried out at 37°C for 3 days. It is confirmed that the only one colony formed can also grow on an L-agar medium containing 25 µg/ml ampicillin, 25 µg/ml chloramphenicol or 25 µg/ml kanamycin.

A plasmid DNA is isolated from cultured cells of the transformant by the same method as in the isolation of pGA22 in Example 1 (1). The plasmid DNA is digested with restriction endonucleases and analyzed by agarose gel electrophoresis. The plasmid DNA has the structure illustrated as pGH2 in Fig. 3. Since the DNA fragment inserted in pGA22 has the same cleavage sites for restriction endonucleases as the cloned DNA fragment containing Escherichia coli operon [refer to Cossart, P. et al.: Molec. Gen. Genet., 175, 39 (1979)], it is clear that pGH2 has a threonine operon.

(2) In vitro recombination of pCG11 and pGH2

pCG11 and pGH2 are completely digested with BglII and BamHI respectively by the same method as in Example 2 (2). Both digests containing 2 µg each of the plasmid DNAs are mixed. 40 µl of T4 ligase buffer solution, 40 µl of ATP (5 mM), 0.2 µl of T4 ligase and 120 µl of H_2O are added to the whole mixture (200 µl). Reaction is carried out at 12°C for 16 hours. The reaction mixture is extracted twice with 400 µl of phenol saturated with TES buffer solution and subjected to dialysis against TES buffer solution to remove phenol.

(3) Recovery of pEthr1

Protoplasts of Corynebacterium glutamicum LA 201 which is a derivative strain of LA 103 strain and requires homoserine and leucine are transformed using as a donor DNA 100 µl of a mixture of a two-fold concentrated TSMC buffer solution and the ligase reaction mixture mentioned above (1 : 1) in the same manner as in Example 1 (3). The transformants are spread on the RCGP agar medium and culturing is carried out at 30°C for 6 days to regenerate the transformants. Cells grown over the whole surface of the agar medium are scraped, washed with physiological saline solution and subjected to centrifugation. The cells are again spread on a minimum agar medium M1 (pH 7.2) consisting of 10 g/l glucose, 1 g/l $NH_4H_2PO_4$, 0.2 g/l KCl, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 10 mg/l $FeSO_4 \cdot 7H_2O$, 0.2 mg/l $MnSO_4 \cdot (4-6)H_2O$, 0.9 mg/l $ZnSO_4 \cdot 7H_2O$, 0.4 mg/l $CuSO_4 \cdot 5H_2O$, 0.09 mg/l $Na_2B_4O_7 \cdot 10H_2O$, 0.04 mg/l $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 50 µg/l biotin, 2.5 mg/l p-aminobenzoic acid, 1 mg/l thiamine hydrochloride and 16 g/l agar and containing 50 µg/ml leucine. Culturing is carried out at 30°C for 3 days. Colonies formed are subjected to selection on an NB agar medium containing 12.5 µg/ml kanamycin and 100 µg/ml spectinomycin. Three strains selected at random are grown in 400 ml of the NB medium to an OD value of about 0.8. Cells are recovered and the plasmids are isolated from the cells by ethidium bromide-cesium chloride density gradient centrifugation described in Example 1 (1).

40 to 55 µg of plasmid DNA is recovered from each strain.

These plasmid DNAs are digested with restriction endonucleases and analyzed by agarose gel electrophoresis as in Example 1 (3) to determine the molecular weights and cleavage sites for PstI, EcoRI and XhoI. The plasmid obtained from one strain is named pEthr1 and the structure is illustrated in Fig. 3. It is confirmed that pEthr1 has the structure wherein a BamHI fragment containing pGH2 threonine operon is combined with pCG11. One of the remaining strains has the same plasmid as pEthr1 and the other has a plasmid wherein the BamHI fragment containing pGH2 threonine operon is combined at the opposite orientation.

Corynebacterium glutamicum LA 103 strain is again transformed with these plasmid DNAs as mentioned above. As the result, strains which do not require homoserine are obtained with high frequency, about 10^{-3} cell/regenerated cell. All of them are endowed with the phenotypes of the resistance to kanamycin and spectinomycin and have the same plasmid as the donor plasmid characterized by the cleavage pattern for various restriction endonucleases.

Example 5

Cloning of a DNA fragment with insertional inactivation:

The possibility of detecting recombinant plasmids of pCE54 vector by the insertional inactivation of a gene responsible for a drug resistance is investigated. pCG4 isolated from Corynebacterium glutamicum 225-250 (FERM P-5939, ATCC 31830) described in Japanese Patent Application No. 58186/81 by the present inventors in the same manner as in the isolation of pCG2 in Example 1 (1) is used as a donor DNA. pCG4 has a molecular weight of 29Kb and cleaved with EcoRI into 4 fragments.

2 units of EcoRI (product of Takara Shuzo Co., 4 units/ml) is added to 50 µl of an EcoRI reaction buffer solution (pH 7.5) consisting of 100 mM Tris-HCl, 7 mM $MgCl_2$, 50 mM NaCl and 7 mM 2-mercaptoethanol and containing 0.5 µg each of pCE54 obtained in Example 1 and pCG4 mentioned above.

The mixture is allowed to react at 37°C for 60 minutes and heated at 65°C for 10 minutes to stop the reaction. 10 ml of the T4 ligase buffer solution, 10 µl of ATP (5 mM), 0.2 µl of T4 ligase and 30 µl of water are added to the reaction mixture and the mixture is allowed to react at 12°C for 16 hours. The mixture is extracted twice with 100 µl of phenol saturated with TES buffer solution and subjected to dialysis against TES buffer solution to remove phenol. Corynebacterium glutamicum LA 103 is transformed using 100 µl of a mixture (1 : 1) of two-fold concentrated TSMC buffer solution and the ligase reaction mixture mentioned above by the same method as in Example 1 (3), and kanamycin-resistant strains are selected. 50 colonies formed are picked up at random and replica-plated on NB agar medium containing 6.25 µg/ml chloramphenicol, 1.6 µg/ml tetracycline and 12.5 µg/ml kanamycin. Culturing is carried out at 30°C for 3 days to determine sensitivity of the strains. 11 strains are resistant to tetracycline and kanamycin but sensitive to chloramphenicol. The plasmids in 10 strains among the 11 strains are isolated by ethidium bromide-cesium chloride density gradient centrifugation as described in Example 1 (1).

These plasmid DNAs are digested with EcoRI and subjected to agarose electrophoreis. Since all the plasmids have higher molecular weights than that of pCE54 and the increased mass corresponds to the size of either of the four fragments formed by the digestion of pCG4 with EcoRI, it is confirmed that the recombinant plasmid contains these DNA fragments at the EcoRI cleavage site present in the gene responsible for the resistance to chloramphenicol of pCE54.

What is Claimed is:

1. A recombinant vector plasmid which is a recombinant of a DNA fragment containing a gene expressible in a microorganism belonging to the genus Corynebacterium or Brevibacterium and a plasmid autonomously replicable in a microorganism of the genus Corynebacterium or Brevibacterium said recombinant being autonomously replicable in a microorganism of the genus Corynebacterium or Brevibacterium and being detectable by the expression of the introduced gene.

2. The recombinant vector plasmid according to claim 1 wherein the gene is derived from a microorganism.

3. The recombinant vector plasmid according to claim 2 wherein the DNA fragment is expressible in a microorganism of Escherichia coli.

4. The recombinant vector plasmid according to claim 1, 2 or 3 wherein the DNA fragment is derived from a microorganism of Escherichia coli.

5. The recombinant vector plasmid according to claim 3 or 4 wherein the DNA fragment contains a gene responsible for drug resistance or a chromosome gene.

6. The recombinant vector plasmid according to claim 1, 2 or 3 wherein the DNA fragment is derived from a microorganism other than Escherichia coli.

7. The recombinant vector plasmid according to claim 6 wherein the microorganism is selected from the bacterium belonging to the genus Corynebacterium, Brevibacterium, Microbacterium, Staphylococcus, Streptococcus, Pseudomonas, Serratia or Bacillus, yeast and Actinomycetes.

8. The recombinant vector plasmid according to claim 1 wherein the plasmid is derived from a microorganism of the genus Corynebacterium.

9. The recombinant vector plasmid according to claim 8 wherein the plasmid is derived from a microorganism of Corynebacterium glutamicum.

10. The recombinant vector plasmid according to claim 9 wherein the microorganism is selected from Corynebacterium glutamicum 225-57 (ATCC 31808, FERM P-5865), Corynebacterium glutamicum 225-250 (ATCC 31830, FERM P-5939) and Corynebacterium glutamicum 225-218 (ATCC 31832, FERM P-5954).

11. Plasmid pCE54 and derivatives thereof.

12. Plasmid pCG11 and derivatives thereof.

13. Plasmid pCB101 and derivatives thereof.

14. Plasmid pEthr1 and derivatives thereof.

15. Corynebacterium glutamicum containing the recombinant vector plasmid in one of claims 1 to 14.

16. A process of producing a recombinant vector plasmid autonomously replicable in a microorganism of the genus Corynebacterium or Brevibacterium and detectable by the expression of the introduced gene, which comprises inserting a DNA fragment containing a gene expressible in a microorganism of the genus Corynebacterium or Brevibacterium into a plasmid autonomously replicable in a microbial cell of the genus Corynebacterium or Brevibacterium.

Fig. 1

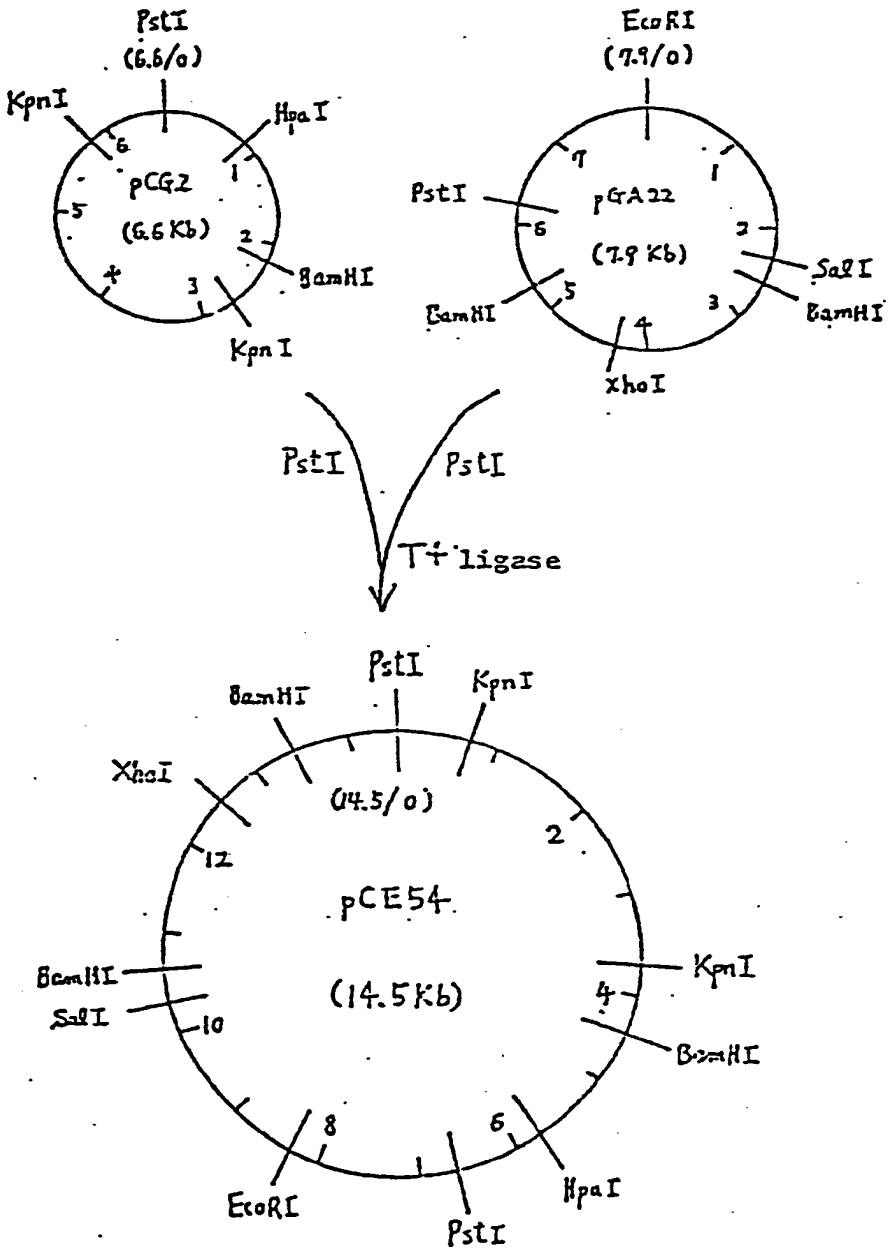


Fig. 2

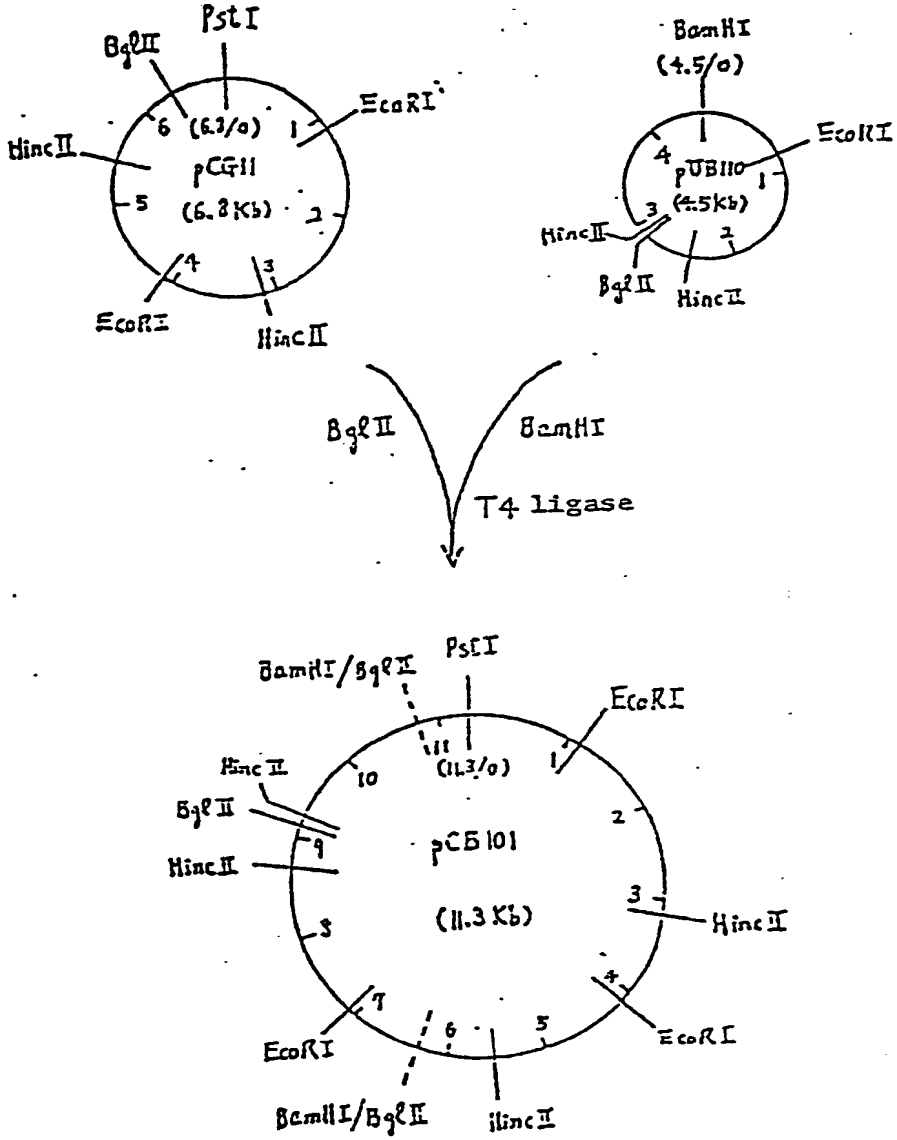


Fig. 3

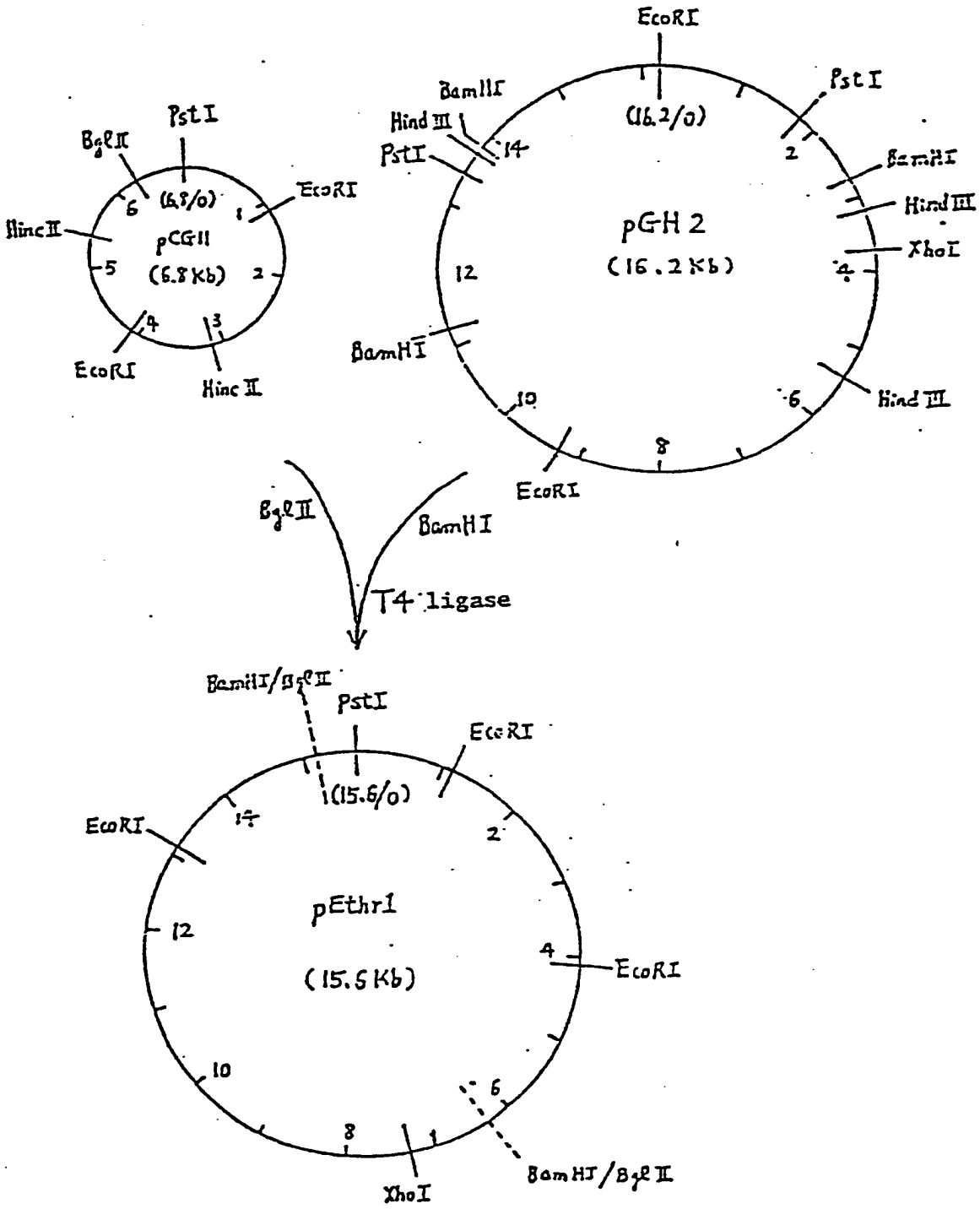
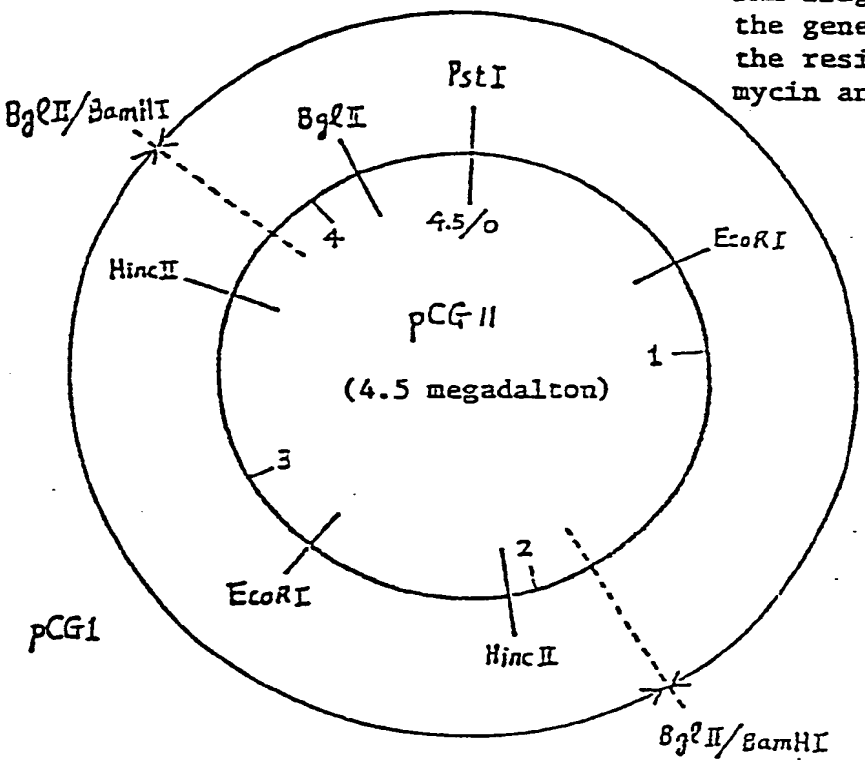


Fig. 4



European Patent Office

Application number 0082485
82111673.8

DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4, OF THE EUROPEAN PATENT CONVENTION

The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

IDENTIFICATION OF THE MICRO-ORGANISMS

Accession numbers of the deposits:

ATCC 31808, FERM P-5865
ATCC 31830, FERM P-5939
ATCC 31832, FERM P-5954